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Short Article

Dap1/PGRMC1 Binds and Regulates Cytochrome P450 Enzymes

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SUMMARY

Cytochrome P450 enzymes are heme-dependent monoxygenases that play a central role in human physiology. Despite the numerous physiological processes that P450 enzymes impact, the electron donors P450 oxidoreductase and cytochrome b5 are the only proteins known to interact with and modulate the activity of ER microsomal P450s. Here, we report that Dap1/PGRMC1 is required for ER P450 function in yeast and humans. We show that *S. pombe* Dap1 is a hemoprotein that binds and positively regulates Cyp51A1 and Cyp61A1, two P450s required for sterol biosynthesis. Similarly, loss of human *PGRMC1* reduces activity of Cyp51A1, blocking cholesterol synthesis and increasing production of toxic sterol intermediates. PGRMC1 stably binds Cyp51A1 and human P450s from three additional families including Cyp3A4, which metabolizes pharmaceutical compounds. These findings demonstrate that PGRMC1 is required for P450 activity and suggest that interindividual variation in PGRMC1 function may impact multiple biochemical pathways and drug metabolism.

INTRODUCTION

Cytochrome P450 enzymes are heme-dependent monoxygenases that participate in the biosynthesis of cholesterol, steroids, bile acids, vitamin D3, and eicosanoids; detoxification of xenobiotics; and metabolism of pharmaceutical drugs (Nebert and Russell, 2002). The human genome codes for 57 cytochrome P450 enzymes, and reduced P450 activity can lead to disease, including congenital adrenal hyperplasia and hypercholesterolemia. Despite the vast array of reactions catalyzed by these enzymes in organisms ranging from bacteria to mammals, the P450 catalytic cycle is remarkably well conserved, requiring one molecule of dioxygen and two electrons from NADPH for the conversion of each substrate molecule to product (Ortiz de Montellano, 2005). Electron donation to eukaryotic ER microsomal P450s occurs primarily through transient interactions with NADPH cytochrome P450 oxidoreductase and, to a lesser extent, cytochrome b5 (Guengerich, 1991; Vermilion et al., 1981; Schenkman and Jansson, 2003). Mammalian mitochondrial P450s interact with the electron-donor proteins adrenodoxin and adrenodoxin reductase (Ortiz de Montellano, 2005). To date, these are the only proteins known to interact with and affect the activity of cytochrome P450s.

The number of P450 enzymes varies widely among species. In contrast to humans and *Arabidopsis thaliana*, which contain 57 and 246 P450 enzymes, respectively, the fission yeast *Schizosaccharomyces pombe* has only two P450 enzymes, Cyp51A1/Erg11 and Cyp61A1/Erg5 (Nelson et al., 2004a, 2004b). These ER-localized enzymes from distinct P450 families are both required for ergosterol biosynthesis: Erg11 catalyzes the demethylation of lanosterol, and Erg5 carries out the reduction of ergosta-5,7,24(28)-trienol (see Figure S1 in the Supplemental Data available with this article online) (Lees et al., 1999). Our recent studies revealed that both *erg11*$^{+}$ and *erg5*$^{+}$ are transcriptionally regulated by the fission yeast sterol regulatory element-binding protein (SREBP), called Sre1, in response to a decrease in oxygen concentration (Hughes et al., 2005; Todd et al., 2006). SREBP is a membrane-bound transcription factor that regulates expression of cholesterol biosynthetic genes and controls lipid homeostasis in mammalian cells (Espenshade, 2006; Goldstein et al., 2006). Importantly, fission yeast Sre1 also controls transcription of cytochrome b5 and cytochrome b5 reductase, raising the possibility that uncharacterized Sre1 target genes may be required for P450 function.

One Sre1 target gene that emerged as potentially having a role in P450 function was the *S. pombe* homolog of mammalian *PGRMC1* and *S. cerevisiae DAP1* (Falkenstein et al., 1996; Hand et al., 2003). Human *PGRMC1* codes for a 22 kDa ER-membrane protein that contains a N-terminal transmembrane segment and a C-terminal cytochrome b5-like domain that binds pentacoordinate heme (Mifsud and Bateman, 2002; Min et al., 2005; Ghosh

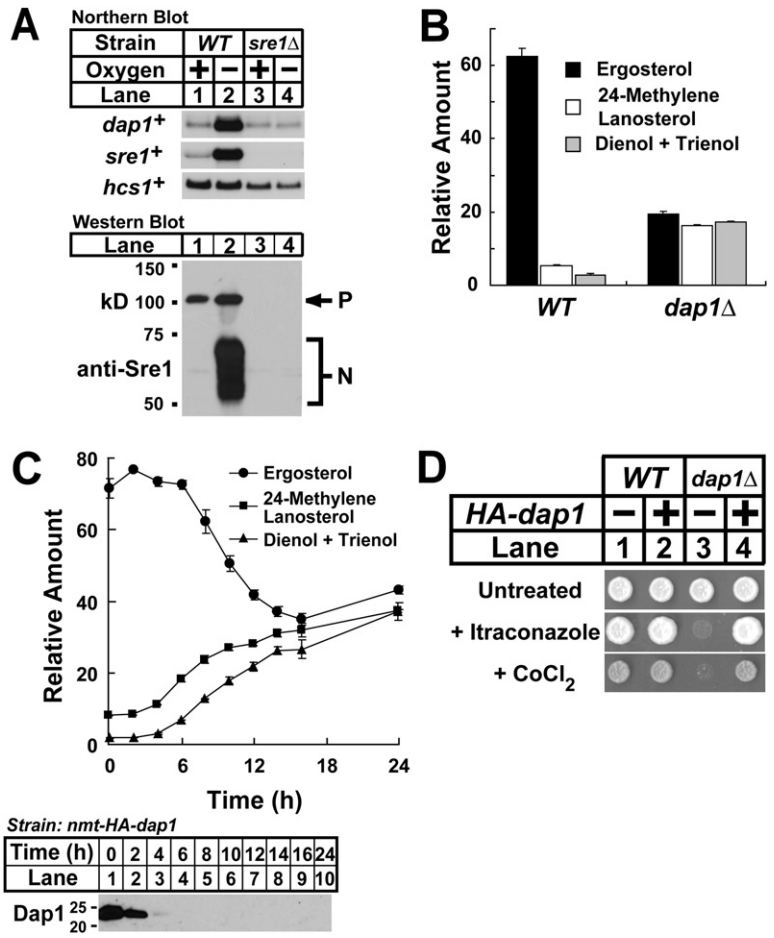

Figure 1. Dap1 Is Required for Sterol Synthesis in Fission Yeast

(A) Wild-type and *sre1*Δ yeast were grown ±oxygen for 6 hr. Total RNA (5 μg) was subjected to northern analysis (top panel), and total cell extracts (50 μg) were subjected to immunoblot analysis with anti-Sre1 IgG (bottom panel). P and N denote the membrane-bound precursor and soluble nuclear form of Sre1, respectively.

(B) Sterols extracted from log-phase wild-type and *dap1*Δ cells (1×10^8 cells) were analyzed by gas chromatography and GC-MS. Cholesterol (5 μg) was added as an internal standard. Data are the average of three independent replicates. Error bars equal one standard deviation. Dienol, ergosta-5,7-dienol; trienol, ergosta-5,7,24(28)-trienol.

(C) At time 0, *nmt-HA-dap1* cells grown in minimal medium lacking thiamine were shifted to thiamine-containing medium to turn off expression of *HA-dap1* from the *nmt* promoter. At the indicated time points, cells were subjected to sterol analysis (top panel), and total cell extracts from the same cultures were subjected to immunoblot blot analysis with anti-HA-HRP (bottom panel). Error bars equal one standard deviation.

(D) Wild-type and *dap1*Δ yeast (5×10^3 cells) containing either no plasmid or a plasmid expressing *HA-dap1* from the constitutive CaMV promoter were spotted on rich medium with no additions, 5 nM itraconazole, or 1.6 mM $CoCl_2$ and grown at 30°C for 3 days.

et al., 2005). Previous studies demonstrated that overexpression of *PGRMC1* in cultured cells increases hydroxylation of progesterone by the cytochrome P450 Cyp21, and monoclonal antibody to PGRMC1 inhibits this activity in rat adrenal microsomes (Laird et al., 1988; Min et al., 2005). In addition, *S. cerevisiae DAP1* is required for normal Erg11 protein levels and ergosterol synthesis in yeast (Hand et al., 2003; Mallory et al., 2005). These data suggest a role for this family of heme-binding proteins in regulation of cytochrome P450 enzymes in eukaryotes.

Here, we present evidence demonstrating a direct role for Dap1/PGRMC1 in cytochrome P450 function. Fission yeast Dap1 is a hemoprotein that binds and positively regulates Erg11/Cyp51A1 and Erg5/Cyp61A1, two P450s required for sterol biosynthesis. Importantly, Dap1 function is conserved in humans. RNAi-mediated knockdown of human *PGRMC1* reduces activity of Cyp51A1, blocking cholesterol synthesis and increasing production of toxic sterol intermediates. PGRMC1 stably binds Cyp51A1 and human P450s from three additional families, suggesting that Dap1/PGRMC1 may function as a general regulator of cytochrome P450s. The presence of *PGRMC1* homologs in plants, flies, and worms suggests that this regulation of cytochrome P450 enzymes may be broadly conserved among eukaryotes.

RESULTS AND DISCUSSION

Our previous microarray analysis revealed that fission yeast *dap1*$^+$ (*SPAC25B8.01*) was coordinately regulated with P450 enzymes by Sre1 in response to low oxygen (Todd et al., 2006). To confirm these results, we examined expression of *dap1*$^+$ in wild-type and *sre1*Δ cells grown in the presence or absence of oxygen. Expression of *dap1*$^+$ mRNA was induced in the absence of oxygen in a Sre1-dependent manner (Figure 1A), consistent with the regulation of other Sre1 target genes such as *sre1*$^+$ itself. HMG-CoA synthase (*hcs1*$^+$) served as a loading control.

To examine whether *dap1*$^+$ is required for ergosterol synthesis in fission yeast, we generated a strain lacking *dap1*$^+$ by homologous recombination and analyzed sterols from wild-type and *dap1*Δ cells using gas chromatography and GC-MS. As observed in *S. cerevisiae*, *dap1*Δ cells showed defects in sterol synthesis (Mallory et al., 2005). *dap1*Δ cells were viable under normal growth conditions but contained a reduced amount of ergosterol and elevated amounts of the ergosterol biosynthetic intermediates 24-methylene lanosterol, ergosta-5,7,24(28)-trienol, and ergosta-5,7-dienol, consistent with defects at the Erg11 and Erg5 enzymatic steps (Figure 1B). Ergosta-5,7-dienol is not a normal pathway intermediate, but

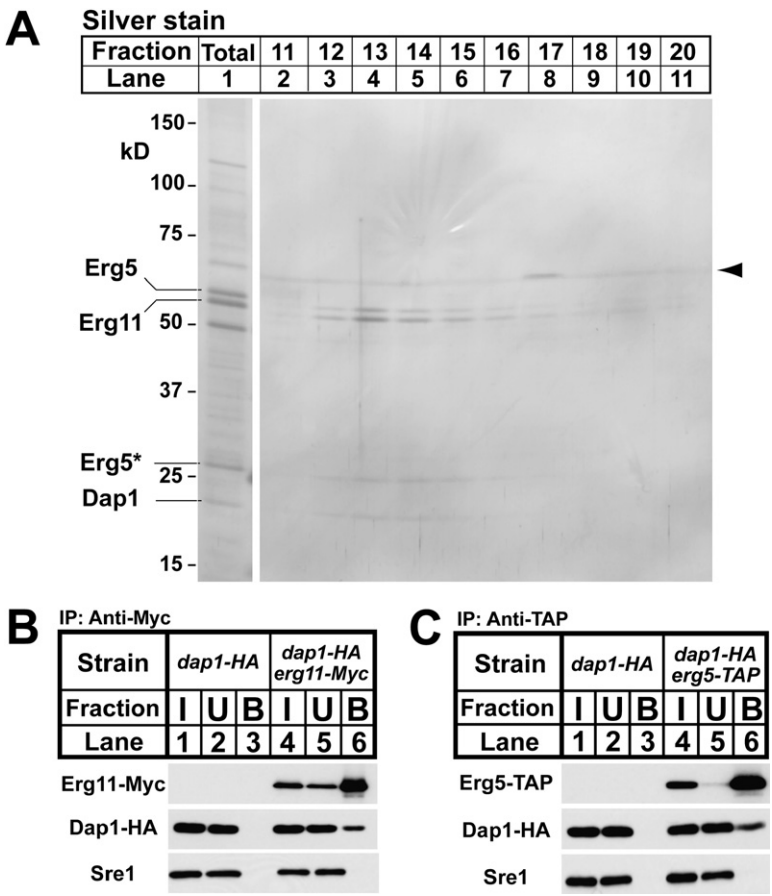

Figure 2. Dap1 Is a Cytochrome P450-Binding Protein

(A) Dap1-TAP was affinity purified from 4 × 10^{10} log-phase yeast cells. Eluted Dap1-TAP protein complexes were subjected to sucrose gradient velocity centrifugation. TCA-precipitated fractions were resolved by SDS-PAGE and visualized by silver staining. Fractions 12–14 from a parallel experiment were pooled and analyzed by mass spectrometry. Proteins that copurified with Dap1-TAP are indicated. * indicates a truncated form of Erg5. Arrowhead denotes keratin contamination.

(B) Detergent-solubilized extracts from *dap1-HA* and *dap1-HA erg11-Myc* yeast were subjected to immunoprecipitation with monoclonal anti-Myc IgG 9E10. Input (I), unbound (U), and bound (B) fractions were subjected to immunoblot analysis with polyclonal anti-Myc IgG, anti-HA-HRP, or anti-Sre1 IgG. Bound fractions are 10-fold overloaded as compared to input and unbound fractions. Sre1 represents the ER-bound precursor form of the protein.

(C) Detergent-solubilized extracts from *dap1-HA* and *dap1-HA erg5-TAP* yeast were subjected to purification with IgG-Sepharose. Input (I), unbound (U), and bound (B) fractions were subjected to immunoblot analysis with rabbit IgG, anti-HA-HRP, or anti-Sre1 IgG.

forms when Erg5 is inhibited (Figure S1) (Skaggs et al., 1996). To determine whether the metabolic flux through these enzymatic steps is reduced as a direct result of Dap1 absence, we generated *nmt-dap1*$^+$, a yeast strain expressing *dap1*$^+$ under control of the thiamine-repressible *nmt1*$^+$ promoter. Addition of thiamine to *nmt-dap1*$^+$ cells resulted in a time-dependent loss of Dap1 protein (Figure 1C, lower panel). Sterol analysis at different times after thiamine addition revealed that, upon loss of Dap1, cells accumulated 24-methylene lanosterol, ergosta-5,7,24(28)-trienol, and ergosta-5,7-dienol, substrates for Erg11 and Erg5. Consistent with this role in sterol synthesis, *dap1*Δ cells are sensitive to the inhibitors of sterol synthesis itraconazole and $CoCl_2$ (Figure 1D). These data demonstrate that Dap1 is required in vivo for the activity of Erg11 and Erg5, the entire complement of cytochrome P450 enzymes in fission yeast. The low sequence identity (23%) between these two enzymes from different P450 families suggests that Dap1 may be a general factor required for P450 enzymes.

To determine whether Dap1 interacts directly with P450s or other sterol biosynthetic enzymes, we purified Dap1 fused to the tandem affinity purification tag, Dap1-TAP. Yeast cells expressing *dap1-TAP* from the endogenous promoter were lysed in detergent, and Dap1-TAP was purified by affinity chromatography. Dap1-TAP protein complexes in the final eluate were subjected to sucrose velocity gradient centrifugation, and gradient fractions were analyzed by SDS-PAGE and silver staining (Figure 2A). Proteins in fractions 12–14 from a parallel gradient were pooled and identified by mass spectrometry (Sanders et al., 2002). These fractions contained three proteins: Dap1-TAP, Erg11, and Erg5. Contaminating proteins in the total eluate did not cofractionate with Dap1-TAP or the P450 enzymes, suggesting that Dap1 binds directly to one or both of these P450 enzymes. To confirm these interactions, we assayed binding of Dap1 to epitope-tagged Erg11 or Erg5 by coimmunoprecipitation. Dap1-HA copurified with both Erg11-Myc and Erg5-TAP, demonstrating that Dap1 forms a complex with both P450 enzymes in fission yeast (Figures 2B and 2C). Binding was specific, as neither Erg11 nor Erg5 bound to Sre1 in the ER membrane. Collectively, these data demonstrate that Dap1 stably binds to Erg11 and Erg5, is required for sterol synthesis, and functions as a positive regulator of two different cytochrome P450 enzymes in vivo.

Dap1 homologs from other organisms are pentacoordinate heme-binding proteins predicted to utilize a conserved tyrosine residue in their C terminus for heme binding (Ghosh et al., 2005; Mallory et al., 2005; Min et al., 2005). To test whether fission yeast Dap1 is a heme-binding protein, we purified soluble recombinant ΔTM-Dap1 lacking the N-terminal transmembrane segment. In parallel, we purified ΔTM-Dap1 Y138F that was predicted not

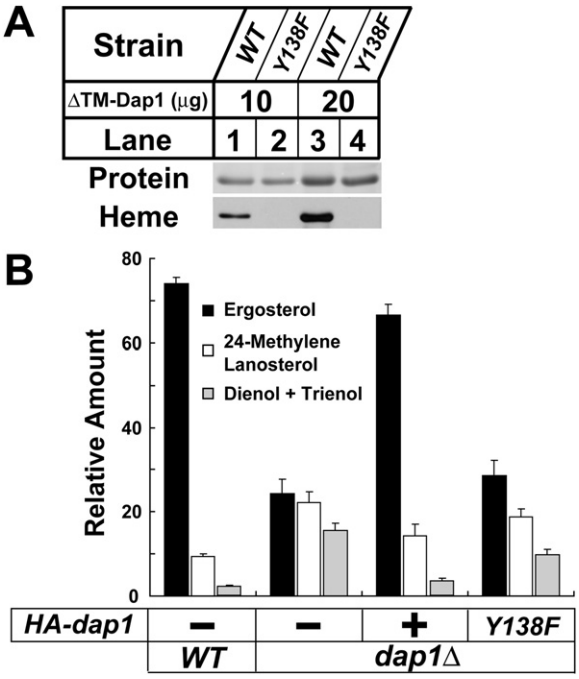

Figure 3. Dap1 Requires Heme

(A) 6xHis-ΔTM-Dap1 protein and the Y138F mutant were purified from *E. coli* and subjected to SDS-PAGE. Protein was visualized by GelCode blue staining (Protein, upper lane) or analyzed for heme content by measuring peroxidase activity in the gel (Heme, lower lane).

(B) Sterols were extracted and analyzed as described in Figure 1B from wild-type and *dap1*Δ yeast containing plasmids expressing *HA-dap1* or *HA-dap1 Y138F* from the CaMV promoter. Data are the average of three independent isolates. Error bars represent one standard deviation.

to bind heme. While both proteins expressed well, in-gel peroxidase assays revealed that Dap1, but not Dap1 Y138F, bound heme (Figure 3A). To determine whether heme binding is required for Dap1 function, we expressed *HA-dap1* and *HA-dap1 Y138F* on a plasmid from the constitutive CaMV promoter in *dap1*Δ cells and examined steady-state sterol levels in these cells. Both wild-type and Y138F mutant Dap1 were expressed at equal levels in yeast (Figure S2). The strain harboring the wild-type *HA-dap1* plasmid showed wild-type sterol levels as anticipated. However, similar to *dap1*Δ, yeast carrying the *HA-dap1 Y138F* plasmid accumulated 24-methylene lanosterol, ergosta-5,7,24(28)-trienol, and ergosta-5,7-dienol, reflecting defects in Erg11 and Erg5 (Figure 3B). These data indicate that *dap1 Y138F* is a loss-of-function mutation and that Dap1 function requires bound heme.

The human genome contains two homologs of fission yeast *dap1*$^{+}$, called progesterone receptor membrane component 1 and 2 (*PGRMC1* and *PGRMC2*). To date, only *PGRMC1* has been characterized (Crudden et al., 2006; Gerdes et al., 1998; Ghosh et al., 2005; Raza et al., 2001; Min et al., 2005). Tissue expression profiles of human *PGRMC1* revealed that it was broadly expressed, with high levels present in liver and adrenal gland, two sites of abundant cytochrome P450 activity (Figure S3). To determine whether PGRMC1 is required for cholesterol synthesis in mammals, we used retroviral infection to generate human HEK293 cells that stably expressed RNA hairpins designed to knock down expression of *PGRMC1* mRNA. Analysis of mRNA and protein levels revealed that the cell line PGR1-1, but not PGR1-2, had reduced expression of *PGRMC1* mRNA and protein (Figure 4A). We performed sterol analysis on HEK293, PGR1-1, and PGR1-2 cells grown in the presence of increasing concentrations of mevalonate to maximize flux through the cholesterol pathway. HEK293 and PGR1-2 cells accumulated the intermediate lanosterol when supplemented with mevalonate (Figure 4B). This level of lanosterol did not affect cell growth after 24 hr (Figure 4B, lower panel). Interestingly, PGR1-1 cells accumulated lanosterol at levels 3-fold higher than control cells. Elevated lanosterol is toxic to cells, and accumulation of lanosterol correlated with the death of PGR1-1 cells at concentrations of mevalonate $\geq$30 mM. No other significant differences in cellular sterols were observed among the cell lines, suggesting that PGRMC1 function was required specifically for the demethylation of lanosterol by the Erg11 human homolog, Cyp51A1. Unlike ergosterol synthesis, cholesterol synthesis only requires one cytochrome P450, Cyp51A1, and thus no homolog of Erg5 exists in mammals. Microsomes from HEK293, PGR1-1, and PGR1-2 cells treated with 0 or 40 mM mevalonate for 10 hr contained equal amounts of Cyp51A1, suggesting that PGRMC1 affected Cyp51A1 activity and not protein stability as reported for *S. cerevisiae* Dap1p (Figure S4) (Mallory et al., 2005).

To test whether PGRMC1 binds to Cyp51A1 in mammals as in yeast, we coexpressed *FLAG-PGRMC1* and *Cyp51-6xMyc* by transient transfection in HEK293 cells. PGRMC1 was quantitatively recovered in coimmunoprecipitations from these cells and the majority of Cyp51A1 copurified (Figure 4C). In a parallel experiment, FLAG-PGRMC1 did not bind Site-1 protease, a membrane protein required for activation of mammalian SREBP, demonstrating the specificity of PGRMC1 for Cyp51A1 (Espenshade et al., 1999). Dap1 bound to both P450 enzymes in fission yeast, yet the amino acid sequence identity between Erg11 and Erg5 is no greater than that between Erg11 and other mammalian P450 enzymes. Consequently, we hypothesized that PGRMC1 may bind to a broad spectrum of P450 enzymes. To test this hypothesis, we assayed binding of FLAG-PGRMC1 to three functionally diverse human P450 enzymes: Cyp3A4, required for xenobiotic metabolism and clearance of ~50% of all known drugs (Rendic, 2002); Cyp7A1, the rate-limiting enzyme in bile acid synthesis (Russell, 2003); and Cyp21A2, a progesterone 21-hydroxylase required for production of glucocorticoids and mineralocorticoids (Ortiz de Montellano, 2005). PGRMC1 bound efficiently to all three proteins (Figure 4D). Collectively, these data demonstrate that PGRMC1 is required for Cyp51A1 activity in cholesterol synthesis and that PGRMC1 may function as a common regulator of cytochrome P450 enzymes in mammals.

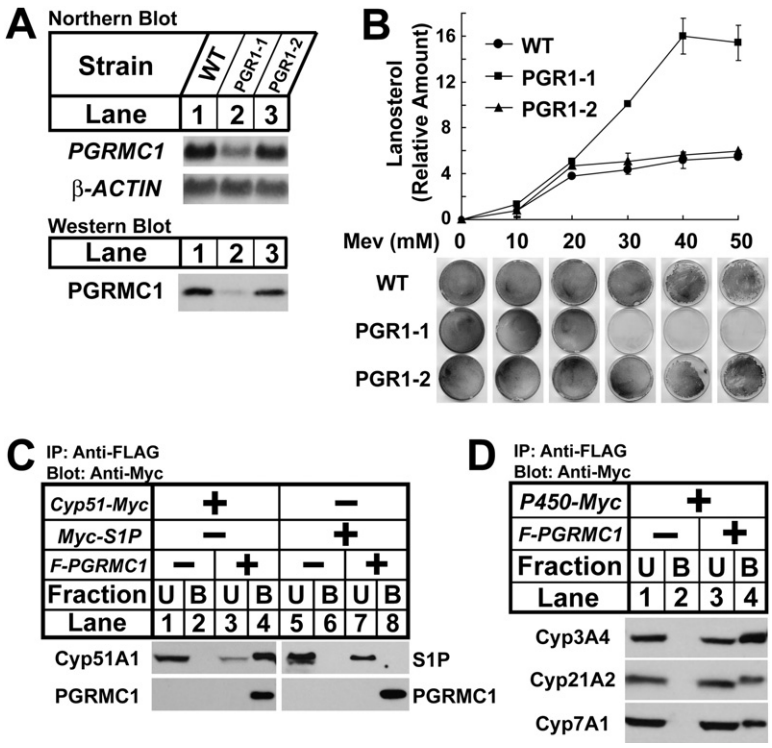

Figure 4. Dap1 Homolog PGRMC1 Is a Cytochrome P450-Binding Protein Required for Cholesterol Synthesis

(A) Total RNA (25 μg) and cell extracts (50 μg) from wild-type, PGR1-1, and PGR1-2 HEK293 cell lines were subjected to northern (top panel) and immunoblot analysis (bottom panel) with anti-PGRMC1 7948 polyclonal serum.

(B) Top panel: On day 0, wild-type, PGR1-1, and PGR1-2 cells were seeded at 5×10^5 cells per well in six-well plates in DMEM + 10% FCS. On day 2, cells were refed with DMEM + 5% lipoprotein-deficient serum (LPDS) and the indicated concentration of mevalonate. After 10 hr, cells were harvested and subjected to sterol analysis by gas chromatography. Ergosterol (5 μg) was added to each sample as an internal standard. Data represent the average of two replicates. Error bars equal one standard deviation. Bottom panel: On day 0, wild-type, PGR1-1, and PGR1-2 cells were seeded at 3×10^6 cells per 10 cm dish in DMEM + 10% FCS. On day 1, cells were refed with DMEM + 5% LPDS and the indicated concentration of mevalonate. After 24 hr, cells were stained with crystal violet.

(C and D) On day 0, HEK293 cells were seeded at 2.4×10^6 cells per 10 cm dish. On day 1, cells were transfected with 2 μg each of the indicated CMV-driven plasmids expressing *FLAG-PGRMC1*, Myc-tagged protein, or empty vector. After 24 hr, cells were harvested, and detergent-solubilized microsomes were subjected to immunoprecipitation with FLAG-agarose. Unbound (U) and bound (B) fractions were subjected to immunoblot analysis with polyclonal anti-Myc IgG or anti-FLAG-HRP. Equal amounts of unbound and bound fractions were analyzed.

Our studies demonstrate that Dap1/PGRMC1 is a conserved hemoprotein required for the activity of cytochrome P450 enzymes in eukaryotic sterol synthesis. Several lines of evidence suggest that Dap1/PGRMC1 has a broad function in cytochrome P450 biology: (1) Dap1 is required for the activity of two P450 enzymes from different families, Erg11(Cyp51) and Erg5(Cyp61); (2) Dap1/PGRMC1 binds to P450 enzymes from five different families; and (3) PGRMC1 has been shown to affect Cyp21 activity in vivo and in vitro (Laird et al., 1988; Min et al., 2005). Known P450-interacting proteins, NADPH cytochrome P450 oxidoreductase and cytochrome b5, supply electrons to cytochrome P450 through transient interactions and are present in catalytic amounts (Estabrook et al., 1971). In contrast, Dap1/PGRMC1 forms a stable, stoichiometric complex with P450 enzymes. Unlike the hexacoordinate heme protein cytochrome b5, Dap1 function requires binding of pentacoordinate heme. This property suggests that Dap1 does not donate electrons to the P450 enzyme. Previous studies hypothesized that *S. cerevisiae* Dap1p may act as a heme chaperone and donate heme to newly synthesized Erg11p because deletion of *DAP1* reduced Erg11 protein levels (Ghosh et al., 2005; Mallory et al., 2005). However, our data favor a model in which Dap1/PGRMC1 is a stable component of a cytochrome P450 enzyme complex and Dap1/PGRMC1 performs an unrecognized, heme-dependent function in the P450 catalytic cycle. Further studies will be required to define both the mechanism and the structural basis of PGRMC1 function. Mutations in cytochrome P450 enzymes cause multiple human diseases, including congenital adrenal hyperplasia (Cyp21A2) and hypercholesterolemia (Cyp7A1) (Nebert and Russell, 2002). Our findings suggest that mutations in *PGRMC1* may lead to more subtle forms of these diseases. In addition, identification of a regulator of P450 enzymes has important implications for drug metabolism research, as polymorphisms in *PGRMC1* may contribute to pharmacogenetic variation observed between individuals, and we are actively investigating this possibility (Evans and Relling, 1999).

EXPERIMENTAL PROCEDURES

Yeast cell culture, immunoblot analysis, northern analysis, and sterol analysis were performed as previously described with minor modifications (Hughes et al., 2005). A complete description of materials, mammalian cell culture, recombinant protein purification, and other methods is available in the Supplemental Experimental Procedures.

Dap1-TAP Purification and Sucrose Gradient Fractionation

The Dap1-TAP purification was conducted as described previously, except that digitonin was substituted for NP-40 in the lysis, wash, and elution buffers (Rigaut et al., 1999). The eluate was subjected to sucrose velocity gradient centrifugation (15%–40% sucrose w/v) to separate Dap1-TAP protein complexes from other nonspecific interacting proteins. Fractions from duplicate gradients were TCA precipitated and analyzed by SDS-PAGE or mass spectrometry (Sanders et al., 2002). Proteins identified in Dap1-TAP protein complexes

were present in the Dap1-TAP total eluate but absent in a duplicate wild-type, untagged yeast eluate.

For coimmunoprecipitations, yeast or transfected mammalian cells were lysed in TAP lysis buffer plus protease inhibitors. Cleared lysates were incubated with antibody and IgG-Sepharose overnight at 4°C and washed with TAP lysis buffer containing 0.1% (w/v) digitonin, and bound protein was subjected to immunoblot analysis.

Retroviral RNAi Production

Retroviruses containing shRNAs homologous to regions in the 3′UTR of human *PGRMC1* mRNA (GenBank accession number NM_006667) were produced according to manufacturer's instructions (Open Biosystems). Briefly, LinX cells seeded at 3×10^6 cells per 10 cm dish on day 0 were transfected on day 1 with 10 μg of either pSM2 Expression Arrest clone v2HS_90636 or v2HS_90640. On day 2, the cells were refed with 8 ml of fresh HEK293 medium. On day 4, the virus-containing supernatant was harvested by filtering and stored at −80°C until further use.

Generation of *PGRMC1* RNAi Knockdown Cell Line

On day 0, HEK293 cells were seeded at 5×10^5 cells per 6 cm dish in 2 ml HEK293 medium. On day 1, cells were infected with 100 μl of supernatant containing retrovirus expressing either *PGRMC1* shRNA clone v2HS_90636 or v2HS_90640 along with a puromycin-resistance marker. On day 2, cells were refed with HEK293 medium supplemented with 3 μg/ml puromycin for selection of cells stably expressing the indicated shRNA. Cells were refed every 3–4 days until only single clones remained. For each shRNA construct, the clones were pooled and expanded to establish two mixed population cell lines, designated PGR1-1 and PGR1-2. PGR1-1 has reduced *PGRMC1* RNA and protein levels and expresses shRNA clone v2HS_90636. PGR1-2, which expresses shRNA clone v2HS_90640, does not have reduced PGRMC1 levels and serves as the negative control RNAi cell line. Both cell lines were maintained in HEK293 medium supplemented with 3 μg/ml puromycin.

Supplemental Data

Supplemental Data include Supplemental Experimental Procedures, Supplemental References, and four figures and can be found with this article online at http://www.cellmetabolism.org/cgi/content/full/5/2/143/DC1/.

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